A Robust and Sensitive Synthetic Sensor to Monitor the Transcriptional Output of the Cytokinin Signaling Network in Planta[1][C][W][OA]

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Cytokinins are classic plant hormones that orchestrate plant growth, development, and physiology. They affect gene expression in target cells by activating a multistep phosphorelay network. Type-B response regulators, acting as transcriptional activators, mediate the final step in the signaling cascade. Previously, we have introduced a synthetic reporter, *Two Component signaling Sensor* (*TCS*)::*green fluorescent protein* (*GFP*), which reflects the transcriptional activity of type-B response regulators. *TCS*::*GFP* was instrumental in uncovering roles of cytokinin and deepening our understanding of existing functions. However, *TCS*-mediated expression of reporters is weak in some developmental contexts where cytokinin signaling has a documented role, such as in the shoot apical meristem or in the vasculature of Arabidopsis (*Arabidopsis thaliana*). We also observed that GFP expression becomes rapidly silenced in *TCS*::*GFP* transgenic plants. Here, we present an improved version of the reporter, *TCS new* (*TCSn*), which, compared with *TCS*, is more sensitive to phosphorelay signaling in Arabidopsis and maize (*Zea mays*) cellular assays while retaining its specificity. Transgenic Arabidopsis *TCSn*::*GFP* plants exhibit strong and dynamic GFP expression patterns consistent with known cytokinin functions. In addition, GFP expression has been stable over generations, allowing for crosses with different genetic backgrounds. Thus, *TCSn* represents a significant improvement to report the transcriptional output profile of phosphorelay signaling networks in Arabidopsis, maize, and likely other plants that display common response regulator DNA-binding specificities.

The plant hormone cytokinin comprises a class of small, adenine-derived organic molecules that influence plant development and physiology in diverse contexts throughout the plant life cycle. Cytokinins initiate a multistep phosphorelay (MSP) signaling cascade by binding to and activating the cognate receptors, hybrid kinases with a cyclases/His kinases-associated sensory extracellular ligand-binding domain (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001). In Arabidopsis (*Arabidopsis thaliana*), these are encoded by the *ARABIDOPSIS HIS KINASE2* (*AHK2*), *AHK3*, and *AHK4* genes. Ligand binding triggers autophosphorylation at a conserved His residue in the receiver domain and subsequent transfer of the phosphoryl group to a conserved Asp residue in the attached transmitter domain. Besides the cytokinin receptors, eight other hybrid kinases are encoded by the Arabidopsis genome, including *CYTOKININ INDEPENDENT1* (*CKI1*), which can potentially activate the MSP signaling network. From the Asp of the hybrid kinase, the phosphoryl group is passed on to one of five ARABIDOPSIS HIS PHOSPHOTRANSFER proteins and then to a nuclear ARABIDOPSIS RESPONSE REGULATOR (ARR), of which there are type-A, type-B, and type-C. Members of the type-B class bind to promoters of target genes via their Myb-like DNA-binding domain and activate transcription, while type-A and type-C ARRs inhibit signaling activity. At the same time, type-A ARRs are immediate-early target genes of activated type-B ARR proteins, which establishes a negative feedback loop to the signaling pathway (Werner and Schmülling, 2009; Argueso et al., 2010; Perilli et al., 2010; Bishopp et al., 2011a; Hwang et al., 2012).

Despite the apparent simplicity of the MSP signaling mechanism, the precise identification and functional characterization of the diverse signaling locales poses several challenges. First, the distribution of active cytokinin ligands in planta is difficult to determine. Cytokinins are produced by complex enzymatic biosynthetic pathways in different cellular compartments and are

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subject to long- and short-range transport and degradation (Werner et al., 2006; Hirose et al., 2008; Bishopp et al., 2011b). Although distribution patterns of cytokinins using monoclonal antibodies have been reported (Aloni et al., 2004, 2005), the available antibodies detect only a subset of active cytokinins, as well as inactive precursor forms (Eberle et al., 1986). Besides AHK2, AHK3, and AHK4, cytokinin-independent hybrid kinases, in particular CKI1 (Pischke et al., 2002; Hejátko et al., 2003, 2009; Deng et al., 2010), but potentially also the ethylene receptor ETR1 (Cho and Yoo, 2007; Hall et al., 2012) or AHK5 (Mira-Rodado et al., 2012; Pham et al., 2012), can activate the MSP network. The use of mutants is complicated because of redundantly acting signaling components, which require the generation of higher order mutants. For many gene families, these are difficult or impractical to generate due to the high number of genes involved, the lack of null mutants, or the close linkage of loci. Moreover, phenotypes caused by a loss of signaling are often pleiotropic or cause early lethality, which can mask functions of interest. In contrast to these difficulties, visualizing the transcriptional MSP output with a synthetic reporter reveals the sites of action during wild-type development. This information then allows focusing on the specific context for functional analyses, such as applying targeted genetic approaches or chemical and pharmacological treatments, and tracking the immediate consequences on the signaling output. The Myb-like DNA-binding domain of the 11 different type-B ARR family members is conserved, in particular, in the nine residues that were shown to make direct DNA contact (Hosoda et al., 2002). Accordingly, in vitro binding studies with the DNA-binding domains of different type-B ARRs identified very similar binding specificities, with the consensus sequence 5′-(A/G)GAT(C/T)-3′ (Sakai et al., 2000; Hosoda et al., 2002; Imamura et al., 2003). This apparent similarity in the DNA binding specificity of the different type-B ARR family members was exploited to design a specific synthetic sensor, *Two Component signaling Sensor* (*TCS*), which is based on concatemeric 5′-(A/G)GAT(C/T)-3′ binding sites (Fig. 1, A–D; Müller and Sheen, 2008). In transgenic *TCS*::*GFP* plants, the *GFP* signal reflecting the signaling output pattern has facilitated describing novel cytokinin functions (Müller and Sheen, 2008; Bencivenga et al., 2012; Marsch-Martínez et al., 2012), as well as refining and deepening the understanding of existing cytokinin functions (Leibfried et al., 2005; Gordon et al., 2009; Zhao et al., 2010; Bielach et al., 2012; Chickarmane et al., 2012; Murray et al., 2012). Despite the documented value of *TCS*-controlled reporters, some limitations emerged, which motivated us to construct an improved version. First, *TCS*-induced expression is weak in certain developmental contexts where cytokinin signaling has a documented role, such as in the embryo sac (Pischke et al., 2002; Hejátko et al., 2003; Deng et al., 2010; Bencivenga et al., 2012), in the shoot (Gordon et al., 2009; Zhao et al., 2010; Chickarmane et al., 2012), and in the vasculature (Mähönen et al., 2000, 2006a, 2006b; Dello Ioio et al., 2008). Second, we observed that GFP expression becomes progressively reduced with increasing generations, such as in the root meristem of the seedling (Fig. 1B), presumably due to silencing effects triggered by the monotony of the repetitive sequence in *TCS* (Chan et al., 2005). Here, we present a superior version, *TCS new* (*TCSn*)::*GFP*, which exhibits higher sensitivity to cytokinin and MSP components in transient transfection assays (Fig. 2) and a much brighter GFP signal in most tissues analyzed (Figs. 3 and 4). Thus, the *TCSn*::*GFP* expression pattern reveals aspects of the MSP output that were not reported by *TCS*::*GFP*. Furthermore, GFP expression has been stable during propagation, indicating that unlike *TCS*::*GFP*, it does not easily get silenced.

RESULTS

Defining Relevant Parameters to Improve TCS Activity

To reliably and consistently monitor low-to-intermediate output levels of the MSP network in planta and to avoid transgene silencing, we sought to improve the current synthetic sensor TCS (Müller and Sheen, 2008). Its design is based on the in vitro-defined DNA consensus sequence 5′-(A/G)GAT(C/T)-3′, as recognized by type-B ARRs (Sakai et al., 2000; Hosoda et al., 2002; Imamura et al., 2003). To identify parameters that affect the activity of TCS, derivatives were constructed with variations in the number of binding sites, phasing, and identity of flanking nucleotides. All of the resulting fragments were cloned upstream of the cauliflower mosaic virus minimal 35S promoter and transcriptionally fused to luciferase (LUC). The ability of these constructs to confer cytokinin-dependent transcriptional activation was experimentally tested in transient transfection assays of primary mesophyll protoplasts (Müller and Sheen, 2008). An oligonucleotide harboring four such bindings sites, separated by arbitrarily selected flanking nucleotides, represented the basic building block for *TCS* (Fig. 1C). Multimerization of this sequence fragment resulted in various derivatives with an increasing number of binding sites (Fig. 1A). The arrangement of binding sites was chosen to realize all possible orientations that two given motifs can have relative to each other: tandem, tail to tail, and head to head (Fig. 1, A and D). Minimal but robust activity was observed with eight binding sites, while 16 sites resulted in a sensitivity comparable to *ARR6*::*LUC*, a reporter based on the 5′ cis-regulatory region of *ARR6* (Hwang and Sheen, 2001), a type-A *ARR* with 11 clustered 5′-(A/G)GAT(C/T)-3′ motifs in its promoter (Fig. 1A; Supplemental Table S2). A dramatic increase in cytokinin responsiveness occurred when the number of sites increased from 16 to 24. Such a sigmoidal response curve is indicative of synergistic interactions among activator binding sites (Carey, 1998). The addition of more sites did not stimulate the activity further (Fig. 1A). Thus, 24 binding sites were chosen for the final design, which was named TCS (Müller and Sheen, 2008). A variant of *TCS*,

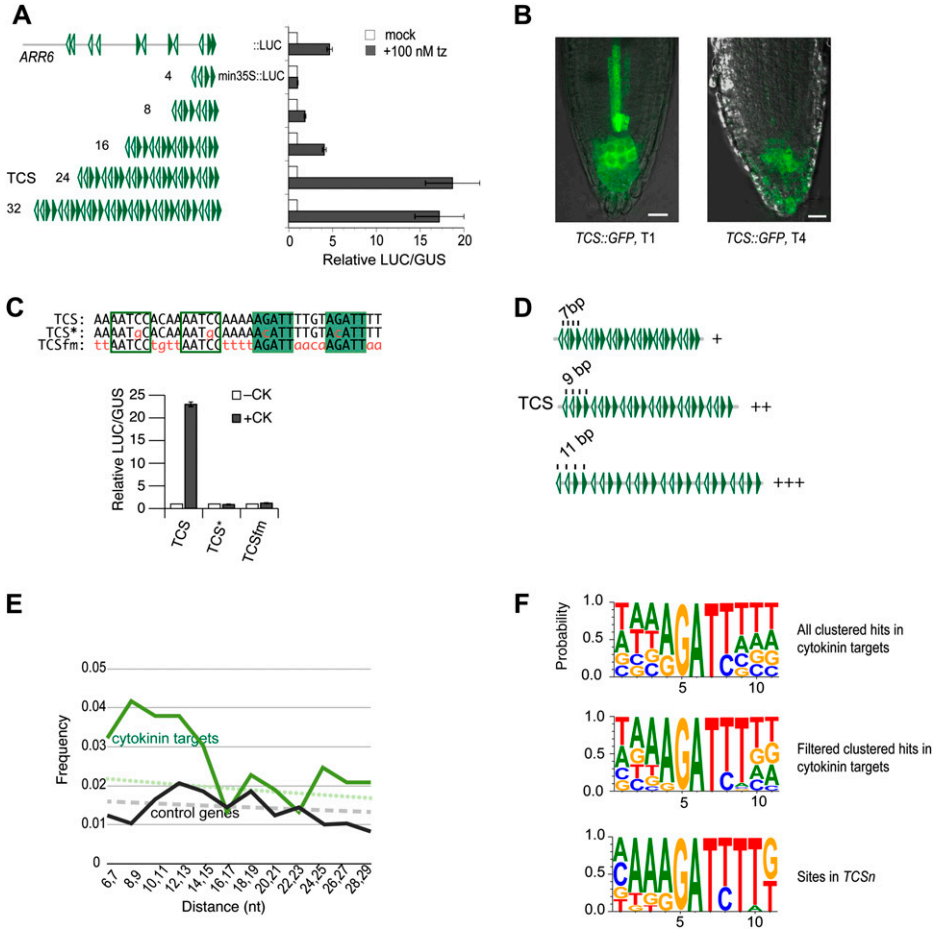

Figure 1. Optimization of *TCS*. A, A concatemer of 24 repeats of the 5′-(A/G)GAT(C/T)T-3′ binding motif caused the strongest cytokinin-dependent induction of a LUC reporter in transient transfection assays. B, Reduced GFP signal in the primary root meristem of a 5-d-old transgenic *TCS*::*GFP* seedling in the fourth generation (T4) compared with a primary transformant (T1). C, Similar to mutating nucleotides essential for in vitro binding of type-B ARRs (*TCS**::*LUC*, where the asterisk indicates point mutation G→C), the mutation of flanking nucleotides (*TCSfm*::*LUC*) abolished cytokinin-dependent response of *TCS*::*LUC*. D, Scheme representing the quantitative effects of different phasings of core 5′-(A/G)GAT(C/T)T-3′ motifs. Phasing of 11 bp results in strongest reporter gene expression. E, The frequency of 5′-(A/G)GAT(C/T)T-3′ motifs with a distance of 7 to 15 bp in cytokinin target genes (top curve) is higher than expected (dotted line), while the same motifs are not significantly higher than expected in control genes (lower curve and dashed line). F, Sequence logos (Crooks et al., 2004) generated from the alignment of clustered 5′-(A/G)GAT(C/T)T-3′ motifs in cytokinin target genes as listed in Supplemental Table S2 (top) after filtering with 5′-A(A/G)GAT (C/T)T-3′ and 5′-A (A/G)GAT(C/T)TT-3′ (middle), and the alignment based on the 12 motifs used to construct *TCSn* as listed in Supplemental Table S4 (bottom). Filled or empty arrowheads (A and D) or boxes (C) indicate 5′-A (A/G)GAT(C/T)TT-3′ motifs on the forward or reverse DNA strand, respectively. Bars = 20 μm. [See online article for color version of this figure.]

TCSfm (for *flanking nucleotides mutated*), harbors mutations in the nucleotides that flank the in vitro-defined core sequences and was expected to integrate cytokinin-dependent induction similar to *TCS*, recapitulating the results of in vitro binding studies. Notably, *TCSfm* is insensitive to cytokinin (Fig. 1C), indicating that the in vitro-defined core motif 5′-(A/G)GAT(C/T)-3′ is too short to support transcriptional activation in vivo. Other derivatives of *TCS* differ in the distance between binding sites (Fig. 1D). Compared to *TCS* with a 9-bp distance between the core motifs, 11 bp resulted in higher activity, while reducing the distance to 7 bp caused a substantial reduction in activity (Fig. 1D). Eleven base pairs correspond approximately to one helical turn of the DNA double helix in its common B configuration (Wang, 1979). In agreement with our findings, helical phasing has been shown to be an important parameter for the functionality of transcription factor binding motifs in individual genes (Bouallaga et al., 2000; Mack et al., 2000; D'Alonzo et al., 2002) and has also been observed at a global scale (Ioshikhes et al., 1999; Makeev et al., 2003). Based on these experiments, we reasoned that improving *TCS* could be achieved by using extended type-B ARR binding motifs and adjusting the phasing of motifs to 11 bp.

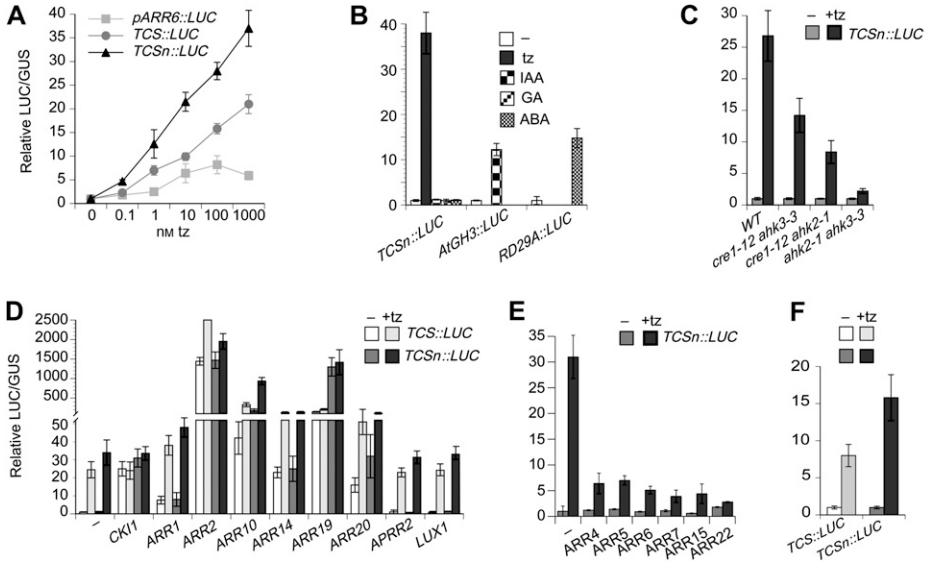

Figure 2. Sensitivity and specificity of *TCSn*::*LUC* in transient transfection assays. A, Induction of *ARR6*::*LUC*, *TCS*::*LUC*, and *TCSn*::*LUC* to increasing concentrations of transzeatin. B, *TCSn*::*LUC* is induced by transzeatin, but not by auxin, GA_3, or abscisic acid. *AtGH3*::*LUC* and *RD29A*::*LUC* serve as positive controls for auxin and abscisic acid hormone induction, respectively (Müller and Sheen, 2008). C, Cytokinin-dependent induction of *TCSn*::*LUC* is compromised in *ahk4 ahk3*, *ahk4 ahk2*, and *ahk2 ahk3* double mutant cells. *cre1-12* is a mutant allele of *AHK4* (Higuchi et al., 2004). D, Positive regulators of the MSP network induce *TCSn*::*LUC* expression. APRR2 and LUX have no effect. E, Type-A and type-C ARR attenuate cytokinin-dependent induction of *TCSn*::*LUC*. F, *TCS*::*LUC* and *TCSn*::*LUC* are induced in maize protoplasts by transzeatin. tz, Transzeatin; IAA, auxin; GA, GA_3; ABA, abscisic acid.

Bioinformatic Analyses to Identify Relevant Type-B ARR Consensus Binding Sites in Vivo

To analyze the type-B ARR binding motifs as they occur in vivo, we analyzed the sequence of the 10 type-A *ARR* genes (*ARR3*–*ARR9* and *AAR15*–*AAR17*). These genes represent the best-documented direct cytokinin target genes (D'Agostino et al., 2000; Taniguchi et al., 2007; Brenner et al., 2012). As a negative control, genes were randomly picked from a list of genes that showed stable expression irrespective of developmental stage, stress, and pharmacological or physiological treatments (Czechowski et al., 2005). Since the cis-regulatory sequence is typically found upstream, but can also be located the within transcribed sequence (Yant et al., 2010; Ritter et al., 2012), about 3 kb of the 5′-upstream sequence, as well as the transcribed sequence from each gene, was included in the analysis, totaling 45 kb of sequence for the 10 type-A *ARR* genes and 58 kb for the control genes (Supplemental Table S6). In each set, the number of hits to the 5′-(A/G)GAT(C/T)-3′ motif was counted. This motif has been shown to be indispensable for type-B *ARR* binding in vitro (Sakai et al., 2000; Hosoda et al., 2002; Imamura et al., 2003) and for function in vivo (Ross et al., 2004; Müller and Sheen, 2008; Zhao et al., 2010; Liang et al., 2012). However, it is short and degenerate and thus occurs frequently by chance: on average, once in 108 bp of random DNA sequence. In the control genes, the frequency of motifs falls within a 95% confidence interval of a Poisson distribution, consistent with random frequency. By contrast, a hit is found every 86 bp in the cytokinin target genes. Such, or even higher, densities are very unlikely to occur by chance assuming Poisson distribution ($P < 10^{-7}$), suggesting that a considerable fraction of the 5′-(A/G)GAT(C/T)-3′ motifs found in cytokinin target genes is functional in integrating cytokinin input (Supplemental Table S1). Next, distances and relative orientations of these motifs were analyzed. Specifically, we measured the distances between two given hits and sorted them into different size classes. The motif itself measures 5 bp; thus, the shortest distance between two hits is 6 bp. Because we were mainly interested in clustered hits, we did not resolve distances greater than an arbitrary 30 bp. The expected size distribution follows the function: $F(n) = (1 - P)^{n-1}P$, where P is the probability to find a hit (Basler, 2000). In the cytokinin target genes, distances from 6 to 30 bp are significantly overrepresented (X^2 [1, n = 527] = 21.1, $P < 0.00001$), suggesting that these motifs are functional to support cooperativity of transcription factor binding in natural promoters, similar to what was observed in synthetic sequences (Fig. 1A). The observed enrichment concentrates to distances of 7 to 14 bp (Fig. 1E; Supplemental Table S3). When the relative orientation of clustered motifs was analyzed, no apparent bias either toward tandem or inverse orientations was detected (Supplemental Table S3). The analysis suggests that clustered 5′-(A/G)GAT(C/T)-3′ hits are

significantly enriched in cytokinin target genes, and we used these hits to create an alignment. An additional three nucleotides flanking the core site were included, totaling 11 nucleotides, which was determined as the optimal phasing based on transient transfection experiments reported above (Fig. 1D). The resulting alignment, represented by a sequence logo (Crooks et al., 2004), revealed the tendency for conservation of nucleotides flanking the core (Fig. 1F). Specifically, sequences accompanied by a 5′ extension, 5′-A-3′ and/or a 3′ extension, 5′-T-3′ appear more frequently, similarly to previous findings (Rashotte et al., 2003; Taniguchi et al., 2007). We used this information to reduce the number of nonspecific motifs from the alignment and filtered the list of motifs using the sequences 5′-A(A/G)GAT(C/T)-3′ and 5′-(A/G)GAT(C/T)T-3′ (Supplemental Table S2). The resulting smaller set of binding motifs yielded a sequence logo with a AA(A/G)GAT(C/T)TT consensus (Fig. 1F). This consensus was also found enriched in the cytokinin target genes compared with the control genes (not shown), similar to previous studies (Brenner et al., 2012).

Based on the refined sequence logo, we created 12 sites, each slightly different from the other. Their alignment creates a sequence logo, which is similar to the natural sites (Fig. 1F; Supplemental Table S4). These synthetic sites were combined in random order to result in a synthetic sequence fragment that was repeated once to harbor 24 binding sites (Supplemental Table S5). Since clustered sites in cytokinin targets show no preference for a specific relative orientation, we preserved the relative site arrangement of *TCS*, which supports all possible orientations (Fig. 1A). This improved synthetic fragment was named *TCSn*. To summarize, the results obtained from transient transfection assays combined with bioinformatic analyses of bona fide cytokinin target genes allowed us to construct a new synthetic cytokinin promoter with an optimized number, spacing, and sequence of motifs, while also including variations to reflect the range of potential diversity among sites found in natural targets. At the same time, sequence variations avoid sequence monotony that could trigger the silencing of *TCSn*::*GFP* in transgenic plants.

TCSn Specifically Integrates MSP Activity

Using transient transfection experiments of mesophyll protoplasts, *TCSn*::*LUC* was subjected to various assays to determine its sensitivity and specificity to MSP signaling (Fig. 2). Its sensitivity to cytokinin was higher compared with *TCS*::*LUC* or *ARR6*::*LUC* (Fig. 2A). Similar to *TCS* (Müller and Sheen, 2008), *TCSn* did not cause transcription of *LUC* upon incubation with the auxin indole-3-acetic acid, GA_3, or abscisic acid (Fig. 2B). Compared with wild-type cells, cytokinin-dependent expression of *TCSn*::*LUC* is compromised in cells that are mutated in two out of the three cytokinin receptor genes *AHK2*, *AHK3*, and *AHK4* (Fig. 2C). Cotransfection with positively acting signaling components, including CKI1 and type-B ARRs, stimulated *TCSn*::*LUC* expression. Notably, overexpression of ARR10, ARR19, and ARR20 caused markedly stronger induction of *TCSn*::*LUC* than of *TCS*::*LUC*, while the remaining type-B ARRs showed similar induction of both reporters. By contrast, ARABIDOPSIS PSEUDO-RESPONSE REGULATOR2 (APRR2) and LUX ARRHYTHMO (LUX), both of which share a very similar DNA-binding domain with type-B ARRs (Hwang et al., 2002; Helfer et al., 2011), do not activate *TCS* nor *TCSn* transcription (Fig. 2D). As expected, cotransfection with type-A ARRs attenuated cytokinin-dependent induction (Fig. 2E). Next, we tested the reporters in maize (*Zea mays*) mesophyll protoplasts. As could be predicted from the similarity of the type-B response regulators between monocots and dicots (Chu et al., 2011), *TCS*::*LUC* and *TCSn*::*LUC*

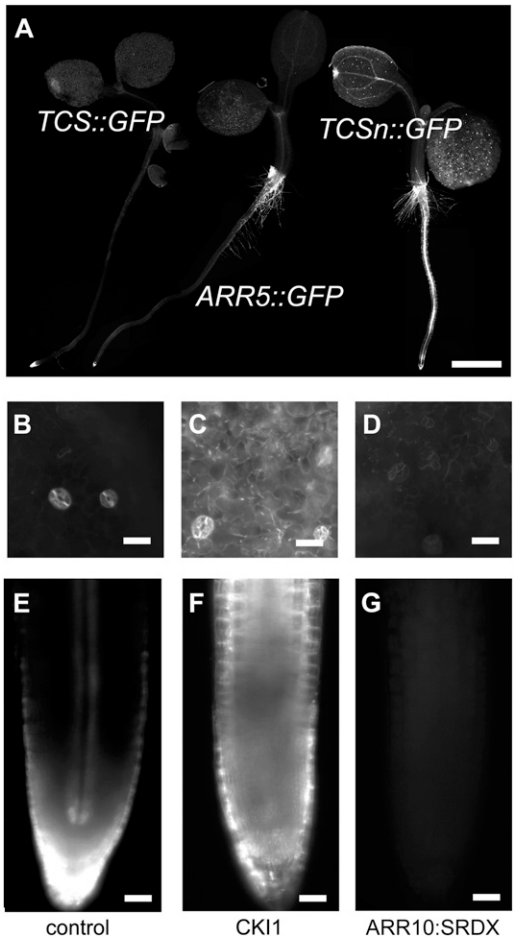

Figure 3. *TCSn*::*GFP* in the seedling. A, Compared with *TCS*::*GFP* and *ARR5*::*GFP*, *TCSn*::*GFP* exhibits strong GFP expression both in the root and shoot of the seedling. B to G, Induced overexpression of CKI1 (C and F) and ARR10:SRDX (D and G) causes ectopic activation or repression of *TCSn*::*GFP*, respectively, compared with controls (B and E) in the cotyledons (B–D) and the root meristem (E–G). Bars = 20 μm.

expression is activated by cytokinin in maize protoplasts (Fig. 2F). In summary, *TCSn* performs superior to *TCS* using protoplast transient assays, while retaining its specificity.

TCSn-Directed GFP Signals in the Seedling Are Brighter Than ARR5- or TCS-Controlled Reporters and Depend on MSP Signaling

Next, we analyzed the GFP expression pattern in plants that were transformed with a *TCSn*::*GFP* construct. Overall, *TCSn*::*GFP* expression levels are higher than *TCS*::*GFP* or *ARR5*::*GFP*, another frequently used cytokinin reporter (Fig. 3A). To address whether *TCSn*::*GFP* expression in planta is also controlled by MSP signaling, we transiently overexpressed proteins that either dominantly activate or repress MSP signaling. Compared with steady mutants, this approach avoids lethality issues and secondary effects. CKI1 induces MSP activity constitutively independent of cytokinins (Hwang and Sheen, 2001; Hejátko et al., 2009). Thus, *CKI1* expression was ubiquitously induced for 30 h in 3-d-old seedlings. Consequently, both in the main root tip and in epidermal cells of the cotyledons, ectopic and ubiquitous signaling activity is observed (Fig. 3, C and F), compared with the control (Fig. 3, B and E). By contrast, *TCSn*-dependent expression is affected by a dominant-negative version of the type-B ARR10. Specifically, ubiquitously expressed ARR10:SRDX caused a loss of the endogenous expression domains (Fig. 3, D and G). This result is in agreement with previous experiments where type-B ARRs harboring a chimeric repressor domain suppress phosphorelay signaling (Heyl et al., 2008; Müller and Sheen, 2008). Thus, ectopic activation of MSP signaling in planta induces *TCSn*::*GFP* expression, while dominant-negative interference with MSP output causes loss of the *TCSn*::*GFP* domains, indicating that *TCSn* specifically integrates MSP output.

TCSn::GFP Expression Patterns Correlate with Known Cytokinin Functions and Also Reveal New Functions

A detailed analysis of the *TCSn*::*GFP* expression patterns in different tissues revealed that the GFP activity is consistent with documented cytokinin functions. For example, during ovule primordia formation, *TCSn*-directed GFP expression, similar to *TCS*::*GFP* (Bencivenga et al., 2012), localizes to the basal part of the funiculus (Fig. 4I). However, while *TCS*::*GFP* expression levels are below the detection level at later stages, *TCSn*::*GFP* expression is visible till female gametophyte stage 7 of ovule development (Fig. 4K). A very weak signal is also detected in the nuclei of the female gametophyte nuclei at the micropylar pole of the embryo sac (Figure 4, J and K, arrows). During embryogenesis, *TCSn*::*GFP* expression is detected in the suspensor and later in the hypophysis. GFP expression is down-regulated in progenitors of the hypophysis defining the basal cell lineage, while a weak signal is detected in the lens-shaped cell, similar to *TCS*::*GFP* (Müller and Sheen, 2008). Notably, expression levels in suspensor and suspensor-derived cells is much weaker compared with *TCS*::*GFP*. By contrast,

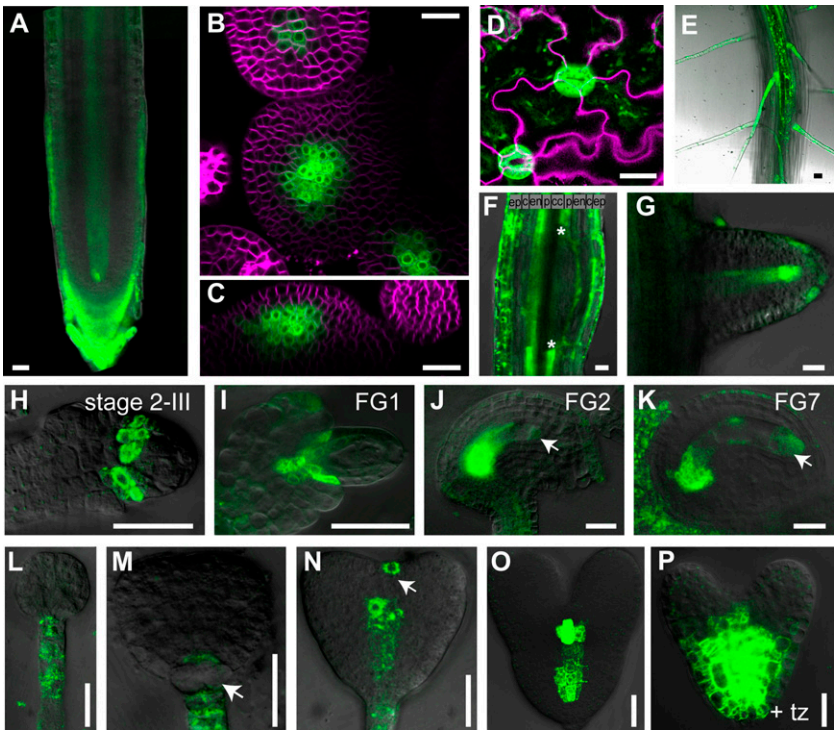

Figure 4. *TCSn*::*GFP* expression in different developmental contexts. A, Primary root meristem of 5-d-old seedling. B, Top view of shoot apical meristem. C, Side view of shoot apical meristem. D, Pavement cells and guard cells. E, Primary seedling root with root hairs. F, Lateral root primordium, early stage. Asterisks delineate lateral root primordium founder cells of pericycle that down-regulate MSP output. G, Emerging lateral root primordium. H, Ovule primordium after first mitotic division of megaspore mother cell stages, according to Schneitz et al. (1995). I to K, Embryo sac, stages according to Christensen et al. (1997). Arrows denote faint GFP signal in nuclei of embryo sac. L to P, Embryos. L, Globular stage. M, Transition stage, arrow denotes down-regulation of GFP in basal cell lineage. N, Heart stage, arrow denotes transient signal in the prospective shoot meristem. O, Late heart stage. P, Late heart stage, overnight incubation with 10 μM transzeatin. The signal from the membrane stain FM4-64 is shown in magenta. tz, Transzeatin; ep, epidermis; c, cortex; en, endodermis; p, pericycle cells; cc, central cylinder. Bars = 20 μm.

provascular cells and the prospective cells of the shoot meristem exhibit distinct and bright GFP signals (Fig. 4, N and O), which potentially allow for addressing novel cytokinin functions in these contexts. As expected, application of exogenous cytokinin leads to increased and expanded GFP expression (Fig. 4P). Expression in the columella cells of the root meristem in the seedling (Fig. 4A), in the vasculature of root (Figs. 3, A and E, and 4A), in the lateral root primordia (Fig. 4, F and G), in root hairs (Fig. 4E), in the shoot meristem (Fig. 4, B and C), in the shoot vasculature (Fig. 3A), and in pavement cells and guard cells (Fig. 4D) are qualitatively very similar to *TCS*::*GFP* (Müller and Sheen, 2008; Bielach et al., 2012; Chickarmane et al., 2012), but much stronger. Thus, in addition to revealing the peaks of cytokinin output, *TCSn*::*GFP* shows intermediate to low levels of signaling output as well. Furthermore, no reduction in GFP levels has been observed after three generations of selfing of transgenic plants (data not shown), indicating that unlike *TCS*::*GFP*, *TCSn*::*GFP* is not subject to transgene silencing.

DISCUSSION

Cytokinins activate a MSP network in target cells, which culminates in transfer of phosphoryl groups to type-B response regulators, nuclear proteins that specifically bind to DNA and activate transcription of selected target genes. Concatemeric binding motifs combined with a minimal promoter and transcriptionally fused with LUC or GFP resulted in a reporter that specifically mediates MSP output in vivo (Müller and Sheen, 2008). Ideally, activity mediated by a synthetic promoter reflects the pure and universal transcriptional output profile of the signaling activity, devoid of tissue-specific aspects or unrelated signaling input. Indeed, *TCS*-dependent expression patterns of GFP or GUS were useful in monitoring the specific sites of phosphorelay signaling output in different tissues, which guided the discovery of previously unknown cytokinin functions (Müller and Sheen, 2008; Bencivenga et al., 2012; Marsch-Martínez et al., 2012) and refined existing models of cytokinin function (Gordon et al., 2009; Zhao et al., 2010; Bielach et al., 2012; Chickarmane et al., 2012; Murray et al., 2012). However, *TCS*-mediated expression in planta is low in many contexts where MSP is known to be important, which motivated us to revise the design of *TCS* by optimizing and extending the binding motifs for the type-B ARRs. Furthermore, to counteract the silencing, we introduced sequence variations in nonessential nucleotides, which broke the monotony of the repetitive *TCS* sequence. These modifications resulted in *TCSn*, which, compared with *TCS*, demonstrates higher sensitivity to cytokinin (Fig. 2A) and a more balanced response to different type-B family members (Fig. 2D).

In transgenic plants, these improvements translate into increased GFP activity in all tissues analyzed (Figs. 3A and 4, A–K, N, and O), except for the suspensor and suspensor-derived cells of the embryo (Fig. 4, L–N). Qualitatively, the expression patterns of *TCS*::*GFP* and *TCSn*::*GFP* in planta are very similar during, for example, ovule primordia formation, embryogenesis, lateral root development, shoot meristem function, and vasculature formation (Figs. 3A and 4). The increased sensitivity of *TCSn*::*GFP* renders additional aspects of phosophorelay readout visible, which remained below the level of detection with *TCS*::*GFP*. Specifically, the expression domain in the shoot meristem is broader (Fig. 4, B and C; Gordon et al., 2009; Chickarmane et al., 2012), a GFP signal in pavement cells becomes visible (Fig. 4D), and a transient signal in the shoot meristem of the embryo at the heart stage is observed (Fig. 4N; Müller and Sheen, 2008). The apparent resistance against silencing will allow the crossing of the *TCSn*::*GFP* line in various genetic backgrounds with a reduced risk of decreased or variable GFP expression in the progeny. This will facilitate the analysis of phosphorelay signaling in many contexts, including the little-known roles in root hair development (Fig. 4E) or the emerging role in pavement (Fig. 4D; Li et al., 2012) and guard cells (Fig. 4D; Desikan et al., 2008; Mira-Rodado et al., 2012), among others.

Ideally, a synthetic reporter integrates the activities of all transcription factors involved in relaying the signal without bias toward specific family members. The 11 members of the type-B ARR family differ in their inherent activity levels, both in transient assays and in planta (Hwang and Sheen, 2001; Sakai et al., 2001; Imamura et al., 2003; Hass et al., 2004; Tajima et al., 2004; Heyl et al., 2008; Müller and Sheen, 2008; Kim et al., 2012; Liang et al., 2012). However, no reference exists for a given family member or relevant combinations thereof, and consequently, it is not clear how the ideal synthetic reporter would respond. Thus, while all type-B ARRs tested activate *TCS* and *TCSn* (Fig. 2D), we cannot exclude that the reporters may exhibit some bias toward specific type-B ARRs. For example, the low expression levels in the suspensor mediated by *TCSn*, compared with *TCS*, might reflect the better binding conditions for the set of type-B ARRs expressed in the suspensor, which facilitates integration of phosphorelay response; while in all other tissues, *TCSn* appears to provide better conditions.

Our experiments using an increasing number of binding elements in synthetic promoters suggest cooperative binding of the type-B ARRs (Fig. 1A). The statistical analysis of cytokinin target genes revealed an enrichment of clustered binding motifs, indicating that cooperative binding occurs in vivo as well. Further support for cooperative binding of type-B ARRs comes from the recent finding that ARR18 can homomerize in planta (Veerabagu et al., 2012). In prokaryotes, DNA-binding response regulators have been shown to dimerize and oligomerize (for review, see Galperin, 2006). Thus, in plants, an analogous model could be realized.

In summary, the improved sensor *TCSn* will allow a detailed study of MSP signaling in various

developmental contexts, shedding new light on plant development and physiology.

MATERIALS AND METHODS

Plant Growth and Treatments

Seedlings were germinated on vertical agar plates containing 1% (w/v) Suc, 0.8% (w/v) phytagar, and one-half-strength Murashige and Skoog medium with a 16-h/8-h photoperiod at 120 μmol m^{-2} s^{-1} and 21°C. After 3 d of growth on vertical plates, seedlings were transferred to liquid medium plates containing 1% (w/v) Suc, one-half-strength Murashige and Skoog medium, 2mM MES, pH 5.7, and 2% (v/v) ethanol for transgene induction in 12-well plates, sealed with parafilm, and incubated for 30 h before recording GFP fluorescence. Plants that needed to be grown to adulthood were kept in the greenhouse with a 16-h/8-h photoperiod at 150 μmol m^{-2} s^{-1} and 21°C during the light period and 18°C during the dark period. Plants used for protoplast transient transfection assays were grown with a 12-h/12-h photoperiod at 90 μmol m^{-2} s^{-1} and 21°C during the light period and 18°C during the dark period. Embryo in vitro cultivation and hormone treatments with transzeatin were performed as described (Müller and Sheen, 2008).

Plant Materials and Reporter and Effector Constructs

The *TCS*::*GFP* reporter line and the expression plasmids used in transient transfection assays were previously described (Hwang and Sheen, 2001; Yoo et al., 2007; Müller and Sheen, 2008). *LUX*, *APRR2*, and *ARR19* coding sequences were cloned into the expression vector as described (Hwang and Sheen, 2001). The *ARR5*::*GFP* reporter is composed of 2.3 kb of upstream regulatory sequence transcriptionally fused to GFP that localizes to the endoplasmatic reticulum (Ottenschläger et al., 2003) in the binary vector pCB302 conferring Basta resistance (Xiang et al., 1999). Plants carrying the inducible transgene *RIBOSOMAL PROTEIN S5A*::*AlcR*/*AlcA*::*ARR10*:SRDX are described in Müller and Sheen (2008). For the *35S*::*AlcR*/*AlcA*::*CKI1* transgene, the DM7 vector conferring kanamycin resistance (Müller and Sheen, 2008) was modified with a ligation-independent cloning adaptor annealed into linearized DM7 vector, digested with *Eco*RI and *Sal*I, to allow ligation of a *CKI1* PCR fragment, amplified from genomic ecotype Columbia DNA, which covers the *CKI1* locus from start to stop codons (see Supplemental Table S5 for sequences of the oligonucleotides). The *TCSn* plasmids *TCSn*::*GFP* and *TCSn*::*LUC* differ from the *TCS* variants (Müller and Sheen, 2008) by the synthetic promoter sequence. Plasmid and oligonucleotide sequences are provided in Supplemental Table S5. All plasmids have been sequenced to ensure no unwanted mutations have been introduced during cloning.

Phenotypic Analysis and Microscopy

GFP expression patterns (Fig. 3) were recorded using a Leica DM6000 microscope equipped with epifluorescence and a Leica DFC350FX camera, or with a Leica SP2 laser scanning confocal microscope (Fig. 4). Micrographs of whole seedlings in Figure 3A were assembled from individual pictures using Adobe Photoshop Creative Suite 4. The lipophilic dye FM4-64 (Molecular Probes) was used at a concentration of 10 μg mL^{-1} to demarcate cell membranes in Figure 4, B to D. Imaging for Figure 4, B and C was done using a Zeiss 510 Meta laser scanning confocal microscope with a 63× air-water dipping lens using the multitracking mode.

Transient Expression in Protoplasts

Protoplast isolation and transfection experiments were performed as reported (Sheen, 1990; Yoo et al., 2007). All protoplast experiments were performed in duplicates, and independent biological replicates yielded similar results.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Summary of 5′-(A/G)GAT(T/C)-3′ hits in cytokinin target genes, control genes, and random sequence.

Supplemental Table S2. List of clustered 5′-NNN(A/G)GAT(T/C)NNN-3′ hits in cytokinin target genes and control genes.

Supplemental Table S3. Distance and orientation of clustered hits in cytokinin target genes and control genes.

Supplemental Table S4. Sites used for *TCSn*.

Supplemental Table S5. Plasmid and oligonucleotide sequences.

Supplemental Table S6. Gene sequences used for the bioinformatic analyses.

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